PORCINE ISLET ENCAPSULATION FOR TREATMET OF TYPE-1 DIABETES USING PRECISION PARTICLE FABRICATION METHOD

BY

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THESIS

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ABSTRACT

Type-1 diabetes (T1D), also known as insulin-dependent diabetes, is characterized by the inability of pancreas to produce sufficient amount of insulin to regulate blood glucose level in the body. Long-term complications such as nephropathy, neuropathy, retinopathy and cardiovascular disease make T1D a major health problem throughout the world. For the past few decades, allotransplantation of insulin-producing islets from the pancreas of deceased human donors has shown promising results in the restoration and sustenance of normoglycemia. Islet transplantation is a suitable procedure, especially for adolescents since it has substantially lower risk in operating procedure than whole-pancreas transplantation. However, only small percentage of patients of T1D can be treated with islet allotransplantation because of the limited donor availability.

Xenotransplantation using porcine islet is a good alternative treatment of T1D. Porcine islet is an ideal substitute of beta cell function for human islet for several reasons: availability, compatibility and functional similarity to human islets. In recent years, treatment with porcine islets has shown significant progress of providing sustained normoglycemia in diabetes-induced nonhuman primates (NHPs), which has demonstrated the feasibility of clinical xenotransplantation. Yet, there are still a number of barriers to overcome in order to accomplish successful clinical application for xenotransplantation.

Harvesting and preserving viable islets with high yield is a first crucial step in porcine islet xenotransplantation. The major limiting factor, however, is the intrinsic fragility of the porcine pancreas that causes various complications in obtaining sufficient amount of healthy islets. Early inflammatory responses and rejection are also the major

problems for clinical xenotransplantation. Immunosuppressive drug is used to regulate the inflammation yet the drug itself in long term induces toxicity in the body. Securing the islets with appropriate encapsulation technology is crucial as it ensures the prolonged viability and function of the islets while avoiding immune reaction from the host after the transplant.

In the first part of the thesis, we demonstrate the isolation and purification method composed of three key components: enzymatic digestion, mechanical disruption and density gradient purification. Porcine pancreata were procured from young commercial breed pigs from the university slaughterhouse. Average time consumption of single islet isolation with the modified procedure is 60 min. Islet yield, purity, viability, *in vitro* function, and morphology were assessed after the isolation. The resulting islet viability and purity were comparable with those achieved in published literature. Clinically relevant yield and quality can be obtained with the use of standard laboratory equipment, which makes this method time- and cost-effective.

The second part of the thesis presents single-step fabrication of core/shell alginate microcapsules using precision particle fabrication (PPF) system to demonstrate the feasibility of successful clinical xenotransplantation. PPF system has following capabilities: precise control of capsule size, ability to process material with high viscosity, high throughput and prevention of cell protrusion. These advantages allow only minimal intervention during the fabrication process, thus making the system suitable for various cell delivery applications including islet encapsulation. Islets with appropriate concentration (in IEQ) for clinical applications were encapsulated in the core of the microcapsules with PPF system. The average duration of microcapsule fabrication

process was 10 min. Islet morphology and viability were assessed immediately after the encapsulation. The intracapsular environment of the alginate microcapsules induces cell-cell and cell-matrix interactions, thus providing hospitable environment for the encapsulated islets. The results showed islets with high survivability after the entire encapsulation process. Future study aimed for improved viability and function of the islets *in vivo* can further support the rationale of the present method as alternative therapeutic option for T1D patients.

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CHAPTER 1

INTRODUCTION

1.1. Background

The International Diabetes Federation (IDF) stated diabetes as one of the largest global health emergencies of the 21st century. As of 2015, there are 415 million people suffering from diabetes worldwide and the number will be increased up to 642 million by year 2040 (http://www.diabetesatlas.org). Type 1 diabetes (T1D) accounts for 10% of these cases. Progressive immune destruction of insulin-producing beta cells is the major indication of T1D which causes various secondary complications such as nephropathy, neuropathy and retinopathy [1]. Insulin injection is a common method of treatment for T1D; however, the process is cumbersome, as it requires constant daily insulin injections, dietary restrictions and monitoring of blood glucose level of the patients. Furthermore, it is difficult to use insulin therapy to permanently maintain blood glucose level within physiological range. Since the fundamental reason for T1D is the loss of functional beta cells in pancreas, a cure for patients with T1D involves the use of replacement islets containing beta cells with the ability of sensing blood glucose level and secreting appropriate amount of insulin [2, 3].

Pancreas allotransplantation and islet allotransplantation are currently the most effective and reproducible therapies to achieve normoglycemia [4-7]. The goals of the allotransplantation are to reestablish long-term glucose-regulated insulin secretion, alleviate the progression of secondary complications of T1D, and improve quality of life [8]. Experimental human islet transplantation has been shown to be effective in reversing

hyperglycemia in rodents, canines [9] and primates [10]. Ever since the first successful whole pancreas allotransplantation to T1D patients in 1966 [11], there has been many experimental clinical trials conducted using deceased organ donors. Pancreas allotransplantation is now widely accepted as therapeutic modality for T1D [12, 13]. However, only selective number of transplantations can be performed due to the limited supply of human donors. Average of 1,200 cases are performed annually in the United States [14]. Difficult surgical procedures and complications after transplantation such as graft thrombosis, pancreatitis, pancreatic fistulae and pseudocyst formation are also the major disadvantages of whole pancreas transplantation [15].

Pancreatic islet transplantation has substantially low risks compare to whole pancreas transplantation, which makes it more accessible to patients with all age groups, especially for adolescents [16, 17]. The procedure involves the selective transplant of islets, isolated from the pancreas of the donors, into the portal vein of the liver, therefore less invasive and has minimal or no complications after transplant [18]. First successful clinical trial was held in 2000 by Shapiro, et al., [19] with the use of the Edmonton Protocol where all seven patients quickly attained insulin independence after transplantation and sustained for medium duration of 11.9 months. Since then, islet transplantation with the use of the Edmonton Protocol has expanded worldwide as a well-established proof-of-concept for T1D treatment [20-22]. Despite the cases of long-term success and rapid progression, however, there are two major barriers that hinder the overall efficiency and effectiveness of islet allotransplantation in clinical application.

The first limiting factor is donor availability. At least two cadaveric pancreas donors are typically required for one islet transplantation which leads to the problem of

mismatch between donor supply and patient demand. There has been search for alternative source of islets which can compensate for the high demand as well as match the criteria for clinical trials. Porcine islet is currently viewed as an ideal substitute to human islet for following reasons: (1) availability; (2) physiological compatibility between porcine insulin and human insulin; (3) possibility of genetic modifications for improved compatibility for clinical applications and (4) ethical regulation conditions [23-25]. Tissues from pigs are proven to be safe and have been used in several clinical applications. Porcine insulin, with only one amino acid difference to human insulin, already has been widely utilized in the treatment of T1D [26].

The second limiting factor is immune-related complications. Islets from an exogenous source, such as porcine islets, are heterologous to human recipients. Although islet xenotransplantation has been utilized in many clinical studies, the recipients had to take immunosuppressing agents to prevent graft rejection and maintain long-term normoglycemia without graft loss. Chronic immunosuppressive therapies can be toxic to the transplanted islets and to other organs of the recipients [27-29]. Immunoisolation using encapsulation technology is a strategy in which islets can be protected from host's immune system after the transplant without immunosuppressive therapy. The strategy involves entrapment of islets in a biocompatible micro-barrier with selective-permeable membrane which can protect islets from early or chronic immune responses, while allowing influx of oxygen, nutrients and ,most importantly, outflow of insulin secreted from entrapped islets [30]. Several studies demonstrated the effectiveness of microencapsulation of porcine islets using various biocompatible materials such as

alginate and agarose gels to reverse diabetes in rodents, canines and non-human primates with the absence of immunosuppressive agents [31-34].

Cell viability and function should be maintained during the encapsulation process. Furthermore, high throughput should be allowed to shorten the intervening period between hospitalization and transplantation.

1.2. Objectives and Contributions

The aim of this study is to demonstrate the porcine islet isolation and purification using modified Ricordi method involving collagenase digestion and Ficoll density gradient purification in order to obtain porcine islets with high yield and high purity. Furthermore, this study provides the feasibility of using precision particle fabrication (PPF) system for clinical xenotransplantation of encapsulated porcine islets using single-step fabrication of core/shell alginate microcapsules.

CHAPTER 2

ISOLATION AND PURIFICATION OF PORCINE ISLETS

2.1. Literature Review

This chapter reviews the current progress of porcine islet isolation and purification methods to emphasize the significance of the three steps: collagenase digestion, mechanical disruption and Ficoll density gradient purification.

2.1.1. Porcine Islet Isolation & Purification

Xenotransplantation using pig as main islet source is currently the most favorable alternative to islet allotransplantation in T1D treatment for following reasons: (1) unlimited supply of islet source; (2) anatomic and physiological similarity of porcine pancreas with human pancreas and (3) advanced progress for possible clinical applications [28, 35, 36]. There are many experimental studies that demonstrated the feasibility of using porcine islets in clinical applications by showing success in restoring normoglycemia of diabetes induced non-human primates for over 6 months [10, 21, 31, 37, 38]. However, the fragility of porcine pancreas makes the overall isolation and purification procedure difficult and inconsistent in outcome [39, 40].

Obtaining healthy islets with high yield is the first crucial step for porcine islet xenotransplantation. These are the following factors affecting the quality and quantity of isolation and purification outcome: (1) physical characteristics of donor pigs; (2) procurement environment and (3) isolation and purification techniques [41].

2.1.1.1. Physical Characteristics

Physical characteristics of donor pigs include age, sex, and strain. One has to consider the optimum condition for each physical aspect in order to secure porcine islets with high yield and function for clinical xenotransplantation.

Numerous studies have shown the differences in xenotransplantation outcome of fetal, neonatal, and adult porcine pancreata. Although there have been reports about feasibility of achieving normoglycemia in non-human primate models in fetal, neonatal and adult porcine islets, there is no conclusive evidence to decide which age of porcine islet source is optimal with regard to porcine islet xenotransplantation [10, 21, 30, 42].

In the early embryonic to neonatal stage of development, which is up to 30 days after birth, insulin-positive cells start to form into islet-like cell clusters (ICC) [43]. The majority of the ICCs consists of epithelial precursor cells, with the absence of definite connective tissues or vasculature, and only less than 40% of the cell clusters are differentiated into endocrine cells including insulin-producing beta cells [44]. Since pancreas has no rigid structural matrix to protect the cluster of insulin-positive cells, the isolation procedure for fetal and neonatal pancreata is known to be simple as it does not require thorough enzymatic digestion and purification process [45, 46]. However, the average yield of ICCs is reported to be lower than the islet yield from adult pancreata and it requires 5-7 days of tissue culture for selective survival and maturation of ICCs into glucose-responsive islet cells [47].

Pig donors with the age above 6 months are categorized as adult pigs. Matured porcine pancreata contains islets with rigid structural matrix and definite connective tissues and vasculature. The average islet yield from adult pigs is significantly higher

than from neonatal or fetal pigs [40, 48, 49]. Immediate graft function against hyperglycemia after transplantation is also one of the major advantages of using adult porcine islets. According to the previous studies, sufficient amount of functional islets can be procured from a single adult porcine pancreas to perform one or two xenotransplantation to non-human primates [50]. Despite high yield and immediate functionality, there are some disadvantages of using adult porcine pancreas such as sensitive immunogenicity, difficult isolation procedure, cost and inconsistent isolation outcomes [39, 51-53]. Nevertheless, adult pig has been recognized as the major donor source for clinical islet xenotransplantation.

There is no definitive result showing which sex/gender is better in terms of the quality of islet isolation outcome. Some studies favored retired female breeders as the isolation results showed procurement of islets with high yield and good compact morphology [40, 48, 49], while other studies suggested using uncastrated male adult pigs with favorable isolation yield [41].

Several studies have reported that choosing the right breed or strain of pigs, especially for young and adult pigs, have significant impact on the yield of islet isolation [54-56]. Study by Kirchhof et al. [57] showed that German Large White (GLW) and German Landrace (GL) have pancreas with more islets per area than Pietrain (PI), Duroc (DU), and Hampshire (HA). Ulrichs et al. [58] reported that GL has relatively higher islet volume density (percent endocrine tissue of the total pancreas mass) than PI, DU, hybrid, and wild breeds. In contrast, the study by Heiser et al. [59] showed higher islet yield with PI compared with GL or hybrid pigs. Kim et al. [23, 41] recently achieved very high islet yield with specific-pathogen-free (SPF) Chicago Medical School (CMS) miniature pigs

(now renamed to Seoul National University miniature pigs) which was not achievable by using miniature pigs with different strains [60]. Since the variability of isolation outcomes was considerably high among isolation attempts from different research groups, there is no best option in terms of choosing breed of donor pigs.

2.1.1.2. Procurement environment

In order to secure viable and functional islets while minimizing risk of transmission of infectious agents, it is important to consider the procurement environment [23, 61]. In many previous studies, procurement procedures were conducted at slaughterhouses. Several problems such as non-sterile surgical environment, less control in breeding conditions of donor pigs, and most importantly, exposure of pancreas to long warm ischemic time (WIT) and cold ischemic time (CIT) led to inconsistencies in isolation outcome. [62-64]. It is crucial to minimize WIT and CIT during pancreas procurement in order to prevent inflammation and cell apoptosis, therefore improving the survivability of islets after isolation and purification stage [65, 66]. Successful porcine islet xenotransplantation to non-human primate models were achieved with WIT within 10 min [51].

2.1.1.3. Isolation and purification technique

The islet yield and viability can be drastically changed under different isolation and purification techniques [67]. After the establishment of Edmonton protocol [19] and automated continuous digestion filtration [68], there has been increased number of clinical islet transplantation due to the improved islet yield and purity. The procedure

involves three major steps: (1) enzymatic pancreatic digestion; (2) mechanical pancreatic disruption; and (3) purification of isolated islets [47, 69].

Islets of Langerhans are highly vascularized and widely distributed inside the pancreas. The primary objective of enzymatic pancreatic digestion is to liberate the islets from the surrounding acinar tissues. Collagen is one of the essential components of the extracellular matrix of the pancreas which can be only degraded by selective protease activities [70]. Intraductal injection of collagenase, an enzyme that breaks the peptide bond of collagen, into the pancreatic duct of pancreas had naturally become the standard strategy for enzymatic digestion [71, 72]. Collagenase not only dissociates the exocrine tissues of the pancreas but also affects the islets; therefore, it is important to consider various factors such as different types of enzyme blends, the concentration of the enzyme, and the duration of the digestion in order to optimize the isolation outcome after the enzymatic digestion stage [55, 73, 74]. Many studies have used mixture of highly pure collagenase with neutral proteases and clostripains to stabilize the digestion while effectively dissociate the exocrine tissues within short amount of time [75].

Once enough collagenase is distributed to the internal matrix, the pancreas is then cut into several small pieces and transferred to a digestion chamber for mechanical disruption, which is a process of physically increasing the area of enzymatic digestion, therefore enhancing the dissociation of the pancreatic acinar tissues. Well established methods include agitating the digestion chamber either manually [71, 76] or automatically [68] for improved separation of the islets from surrounding acinar tissues. The islet separation (cleave index) was more effective with the automated method; however the overall procedure and installation are complicated and expansive. Although

manual method has relatively low cleave index, the procedure is much cheaper and easy to handle [77, 78].

The purification step involves extraction of isolated islets from the digestion chamber and removal of pancreatic tissue fragments. Good experience in different purification techniques determines the purity and recover rate of islets after the isolation. Discontinuous density gradient is recognized as the standard method for the islet purification; however the purification outcome has not been consistent amongst the previous studies because the outcome is subjective to one's technique and experience along with ambiguity in assessment [79]. Islet purity from the early studies was less than 40%, which could be explained by the low experience in isolation and purification method in the initial stage of development [79, 80]. More recent studies showed drastic improvement in islet purity as high as 75-95% by substituting different types of gradient solvent or implementing chemical-free filtration methods [41, 81, 82].

The studies by Mirkovitch et al. [83] and Kretschmer et al. [84] demonstrated the significance of islet purification with canine models by experimenting autotransplantation without the purification step. The pancreatic tissue fragments and toxic subcellular particles induced host's immune response, which eventually led to several post-transplant complications such as malnutrition and total pancreatectomy.

2.2. Materials and Methods

2.2.1. Porcine pancreas procurement

4-6 months old Duroc-Yorkshire hybrid pigs from University of Illinois Meat Science Laboratory slaughterhouse were used for this experiment. Two porcine pancreata

were procured, immersed in 4°C Hank's balanced salt solution (HBSS) with 10% FBS and transported to the laboratory. The warm ischemia time (WIT) was 10 min and the cold ischemia time (CIT) was minimized to 30 min. Before initiating the isolation procedure, fat tissues and blood vessels on the surface of the pancreas were trimmed with surgical scissors.

2.2.2. Porcine pancreas isolation

Modified Ricordi method [47] was used to perform islet isolation. In order to use only the splenic lobe, the duodenum lobe and connective lobe of the donor pancreas were removed with surgical scissors. The pancreatic duct of the splenic lobe was located and cannulated to slowly infuse 200 mL of collagenase solution (1 mg/mL collagenase from Clostridium histolyticum in HBSS with 10% FBS, Sigma, St. Louis, MO). After the infusion, the splenic lobe was cut into small pieces and incubated in a shaking incubator at 37°C for 15 min for further enzymatic digestion. 100 mL of 4°C HBSS with 10% FBS was added to the digested tissues to dilute the collagenase solution in order to prevent collagenase from damaging the isolated islets. Stack of three sterile meshes with the diameters of 1.18, 0.71 and 0.595 mm were used to filter the digested tissues. The filtrate containing isolated islets were transferred to 50 mL conical tubes and centrifuged at 1,500 rpm for 2 min at 4°C to remove collagenase.

2.2.3. Porcine pancreas purification

The isolated islets were gently resuspended in 6 mL of 25% (w/v) Ficoll solution in dH_2O (Ficoll 400, Sigma, St. Louis, MO). A discontinuous gradient of 23, 20.5 and 11%

(w/v) Ficoll solutions were slowly layered, respectively, on top of the islet suspension and centrifuged at 2,500 rpm for 10 min at 4°C. The top Ficoll layer, where the majority of the islets congregated after centrifugation was collected to a new 50 mL conical tube. Centrifugation, at 2,500 rpm for 10 min, was used to discard Ficoll solution and wash islets two times with HBSS. The purified islets were resuspended in RPMI-1640 with 10% FBS and 1% Pen/Strep and incubated in water-jacketed CO_2 incubator at 37°C with 5% CO_2.

2.2.4. Islet evaluation

Dithizone (DTZ) staining [85, 86] was used to determine the quantity and quality of the isolated porcine islets. 100 ul of DTZ stock solution (5 mg/ml in DMSO) was added to 1 ml aliquot of islet sample in a calibration grid. After letting DTZ to stain the insulin granules of the islets, red color stained islets were identified and counted using optical microscope. The total quantity (islet particle number, IPN) of the islets was converted to islet equivalents (IEQ) for standardized assessment of islet quantity.

Trypan blue staining [87] and SYTO-green/Ethidium bromide (EB) staining [88, 89] were used, respectively, to quantitatively and qualitatively assess islet viability immediately after the purification. 100 ul of 0.4% trypan blue solution in PBS was added to 1 ml aliquot of islet sample in a calibration grid. Optical microscope was used to quantify the viable cells as a means to assess viability of islets after isolation and purification. In a separate calibration grid with 1 ml aliquot of islet sample, 100 ul of SYTO-green solution (5mM in DMSO) and 50 ul of EB (10 mg/ml) were added

consecutively. Fluorescence microscope was used to separately identify viable and dead islet cells.

In vitro function of the islets was assessed by measuring their dynamic insulin secretory response under low or high glucose stimulation [90]. One group (n=4) of islets was challenged with low glucose stimulation (1.67 mM) while the other group (n=4) was challenged with high glucose stimulation (16.7 mM) for 60 min at 37°C with 5% CO_2. Porcine Insulin ELISA assay kit (Mercodia, Uppsala, Sweden) was used to assess the resulting responses.

2.2.5. Statistical analysis

All data were expressed as mean ± standard deviation (SD). Data comparisons were performed by one-way student t-test. Differences with $p < 0.05$ were considered statistically significant.

2.3. Results and Discussion

2.3.1. Islet yield and morphology

Total of two porcine pancreata of 4-6 months old Duroc-Yorkshire hybrid pigs were used for this experiment. Only the splenic lobe of each pancreas was used as it has shown more consistent isolation results compared to those using whole pancreas [91, 92]. Table 1 shows that two separate operations, with the average splenic lobe weight of 73.0 ± 0.57 g, gave similar islet isolation results with 2,460 ± 404 IEQ/g before the purification and 1,950 ± 526 IEQ/g after the purification which were similar to those reported with the same collagenase source [91]. The average recovery rate and viability

were 78.6 ± 8.49% and 88.9 ± 0.57%, respectively, which were also similar to those reported previously [60]. Figure 1 shows the morphology of the isolated porcine islets before and after DTZ staining. The isolated islets had round or oval shape with distinct golden brown color. Rigid surface structure without any cell protrusion indicates the healthy condition of isolated islets [93]. The size of the islets were varied from 50 um to 250 um which is shown in Figure 2. Enzyme source and Enzymatic digestion duration are reported to be important factors for determining the size distribution and yield of isolated islets [94]. With the procedure presented in this study using crude collagenase, with the exposure time of 20 min, showed the majority of islets (80%) distributed within 50-150 um size range. Large diameter islets (>100 um) with more centralized vascular structure can endure against the damage from the enzymatic digestion and thus have a potential for better viability and function after isolation and purification [53]. However, islets with larger diameter (>150 um) and concentrated core are reported to be more susceptible to hypoxia compared to small size islets because of the difficulty of oxygen supply to the core of the islet clusters [95]. Hypoxia-induced apoptosis causes decrease in islet viability and impairs beta cell function [96, 97]. Bright red color from DTZ staining represents the insulin-containing beta cells inside the islet clusters [85]. Figure 3 visually depicts the viability of the isolated and purified porcine islets. Group of Islets with average viability above 70% is considered to be viable sample [98].

2.3.2. Islet function *in vitro*

Table 2 shows the average glucose-stimulated response of the isolated porcine islets immediately after purification. The isolated islets had average insulin secretion of

3.29 ± 1.51 μU/IEQ/hr at low glucose stimulation (1.67 mM) and 5.88 ± 1.27 μU/IEQ/hr at high glucose stimulation (16.7 mM). The stimulation index (SI), which is a ratio between insulin secretion at high and low glucose stimulation, is a widely used parameter to determine the level of function of isolated islets [99]. The resulting SI for this experiment was 2.01 ± 0.72 which was within the range from the previous studies which was from as low as 1.5 to as high as 2.58 [99, 100].

2.4. Conclusion

In this study, we demonstrated a modified isolation procedure using crude collagenase and purification using Ficoll discontinuous density gradient method [101]. The average yield after purification was 1,950 ± 526 IEQ/g after the purification with the viability of 88.9 ± 0.57%. Establishing a laboratory with an isolation method to preserve viable islets with high yield is a first crucial step for the future work regarding islet xenotransplantation. Isolated and purified islets from two porcine pancreata were sufficient in amount to be used for the microencapsulation using precision particle fabrication (PPF) method.

2.5. Tables and Figures

Table 1. IPN, IEQ, recovery and viability assessment

	Splenic lobe weight (g)	Pre-Purification			Post-Purification			Recovery (%)*	Viability (%)
		IPN	IEQ	IEQ/g	IPN	IEQ	IEQ/g		
Median	73.0	314,000	179,470	2,460	176,800	142,214	1,950	78.6	88.9
SD	0.57	53,740	28,106	404	10,182	37,347	526	8.49	0.57

IPN: islet particle number IEQ: islet equivalents

* Recovery (%) = IEQ (pre-purification) / IEQ (post-purification) x 100

Table 2. Glucose-stimulated insulin secretion (GSIS)

IEQ/g (post-purification)	GSIS (μU/IEQ/hr)		Stimulation index (SI)*
	Low glucose	High glucose	
1,950±526	3.29 ± 1.51	5.88 ± 1.27	2.01 ± 0.72

Stimulation index = high glucose GSIS / low glucose GSIS

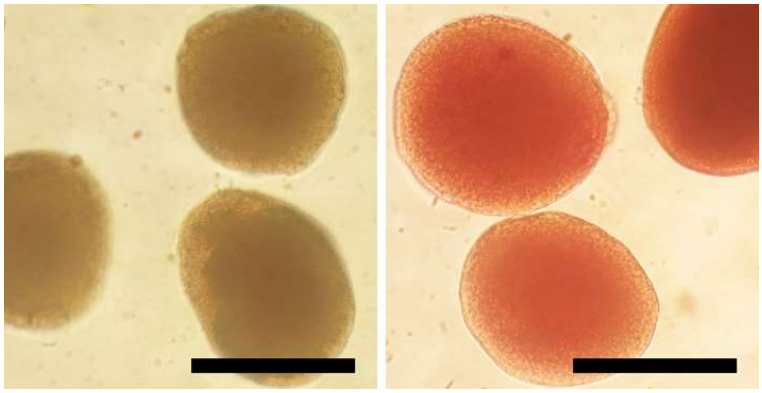

Figure 1. Morphological characteristics before (left) and after (right) dithizone (DTZ) staining of isolated porcine islets. DTZ (red) selectively stains pancreatic beta cells by chelating the zinc ions presented in the insulin granules of the islets. Scale bar = 200 μm.

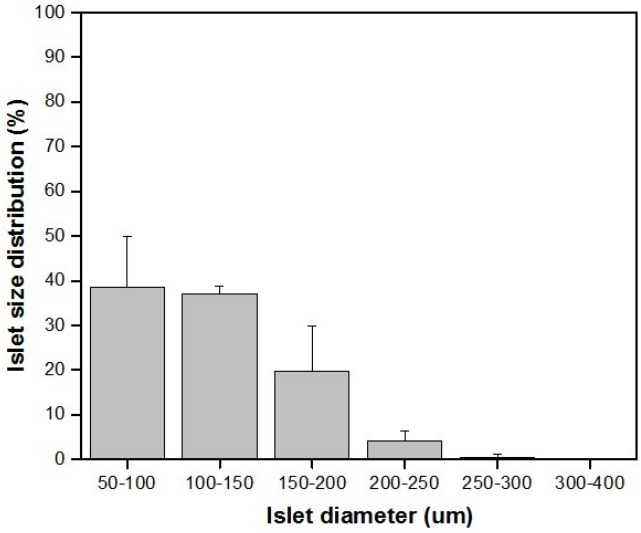

Figure 2. Average size distribution of isolated islets from two porcine pancreata. Data are represented as mean ± SD (n=2).

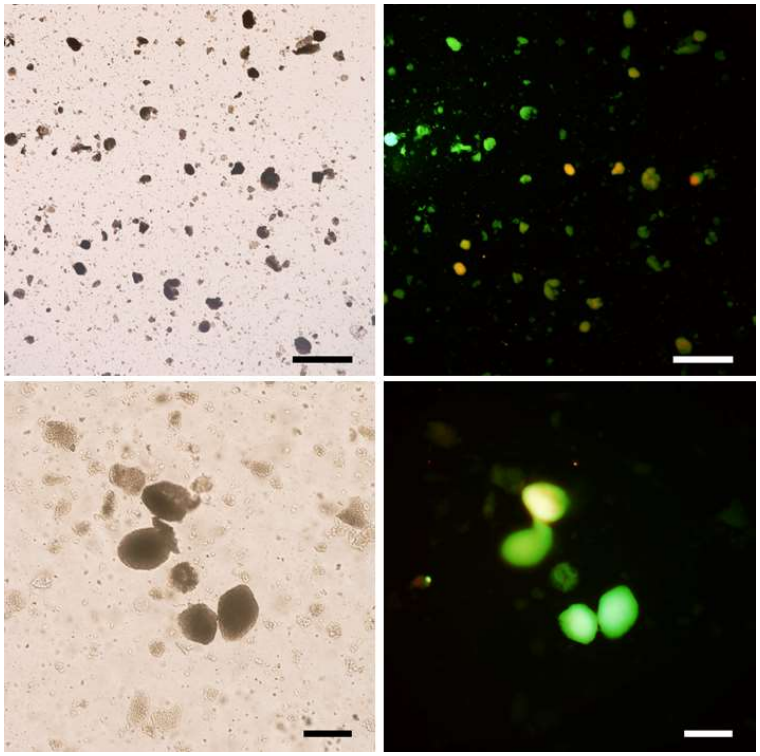

Figure 3. Optical (left) and fluorescence (right) micrographs of isolated porcine islets. SYTO-green staining and ethidium bromide (EB) staining were used to visualize islet viability after isolation and purification. SYTO-green (green) stains viable islets and EB (red) stains dead islets. Scale bar = 500 μm (top) and 200 μm (bottom).

CHAPTER 3

PORCINE ISLET ENCAPSULATION USING PRECISION PARTICLE FABRICATION (PPF) METHOD

3.1. Literature Review

This chapter explains the fundamental concept of applying precision particle fabrication (PPF) system into various cell delivery applications, especially for clinical xenotransplantation of porcine islets for T1D treatment.

3.1.1. Immunoisolation

The rapid progression in the field of islet isolation and purification solidifies the feasibility of clinical islet xenotransplantation; however, achieving long-term success of insulin independence in clinical trials still remains uncertain. Pancreatic islets from xenogeneic source, such as porcine islets, are subject to human recipient's immune reaction, which eventually leads to graft rejection. Most studies demonstrated that $CD4^+$ T cells mainly trigger acute xenograft rejection [102, 103]. Direct contact of transplanted porcine islets with the host's blood triggers instant blood-mediated inflammatory reaction (IBMIR) which causes many complications such as coagulation, islet-membrane leakage and antibody deposition [104, 105]. Therefore, the current clinical therapy requires the use of anti-rejection medication, also known as immunosuppressive medication, to achieve chronic immunosuppression which is to protect transplanted islet grafts from host's immune reactions. However, the prolonged exposure to even the most optimized

medications is known to be toxic to both transplanted islets and recipient's own organs [28, 106].

One approach to prevent post-transplant rejection is to use immunoisolation strategy of encapsulating islets with immuno-protective biomaterial [107]. The semi-permeable membrane formed by the biomaterial serves as an immuno-barrier that protects the encapsulated islets from the host's immune system, thereby allowing islets to regulate blood glucose level for a longer period of time [108, 109].

3.1.2. Microencapsulation

Ever since the first implementation, which is now categorized as first-generation drug delivery system, in 1952 by the pharmaceutical company Smith Kline & French [110], the concept of controlled-release drug delivery system has been widely studied and utilized in various drug and live cell based treatment. The first-generation (1950-1980) was mainly focused on establishing sustained release therapy by controlling the release kinetics of the injected drug. The mechanism is based on drug's behavior to dissolution, diffusion, osmosis and ion exchange [111]. The first-generation delivery methods are still commonly used for producing 'once or twice a day' delivery system. Despite the success of the first-generation delivery system, there has been an increasing attempt of developing more advanced delivery system to achieve higher efficiency and safety, which naturally led to the beginning of the era of second-generation delivery system (1980-2010). The development of micro- and nanotechnology has triggered the development of various drug encapsulation strategies using smart polymers and hydrogels [111, 112]. Micro- and nano-encapsulation with attractive properties showed the following

advantages in the field of drug delivery system: (1) an effective protection of the encapsulated agents from damage from enzymatic degradation or immune reaction; (2) more accurate controllability in drug release kinetics based on environmental factors; and (3) easy administration with less dosage frequency [113]. Microcapsules, or microbeads, can serve as reservoir of all sorts of substances in solid or liquid form and are, therefore, suitable for delivering various active agents such as drugs, proteins, bacterial cells and stem cells. The well-defined barrier formed by the microcapsules can safely protect the encapsulated agents from host's immune reactions, thus allowing more controlled and sustained release [114, 115]. Number of studies has shown the immunoprotection capability of microcapsules in Parkinson's, Alzheimer's, and type 1 diabetes (T1D) [116-119]. Additionally, the semipermeable membrane with porous surface of the microcapsules allows influx of essential molecules such as nutrients and oxygen to maintain cell survival, and efflux of therapeutic agents such as insulin to induce sustained treatment of the target disease [120, 121].

3.1.3. Biomaterial: Alginate

Among the list of many biomaterials, alginate has emerged as one of the most extensively explored in drug delivery applications due to the following appealing characteristics: (1) good biocompatibility and cytocompatibility; (2) control in biodegradation; and (3) versatility in modifications [122, 123]. In addition, and most importantly, alginate dissolved in aqueous solution can be ionotropically crosslinked using divalent cations such as calcium (Ca^{2+}), barium (Ba^{2+}) and strontium (Sr^{2+}), which instantly convert alginate solution into 3D matrix called hydrogel. Fast crosslinking is

one of the strongest suits that make alginate the most suitable biomaterial to be used in encapsulation of sensitive or delicate bio-agents such as proteins, genes and living cells [124-126].

3.1.4. Precision Particle Fabrication (PPF) System

There are many methods to prepare alginate microcapsules which can be divided into three categories: chemical, physiochemical, electrostatic and mechanical processes [113]. One needs to consider how to provide required amount of therapeutic agents at the given amount of time to the target location to optimize the efficacy and minimize side effects [127]. Precision particle fabrication (PPF) system pioneered by Kyekyoon (Kevin) Kim [128, 129], which uses acoustic excitation to mechanically generate uniform microdroplets from a liquid jet containing polymer solution, has the following appealing capabilities: (1) precise control of microcapsule size and uniformity; (2) handling of polymer solution with high viscosity; (3) prevention of protrusion of inner content by forming core/shell structure; and (4) high throughput and rapid process [127]. Alginate in aqueous solution is highly viscous, therefore difficult to handle with conventional fabrication method. However, PPF system with its acoustically excited pressure can break a liquid jet, consisting of alginate solution, into uniform microdroplets in desired size. The dual nozzle allows PPF system to generate microdroplets with core/shell structure in order to safely immobilize various cell sources, such as porcine islets, inside the microcapsules, thereby being able to achieve immunoisolation from host's immune cells.

As a novel cell encapsulation method, PPF system was used in this study to produce semi-permeable alginate core/shell microcapsules encapsulating porcine islets to further demonstrate the potential of islet transplantation in the treatment of T1D.

3.2. Materials and Methods

3.2.1. Islet encapsulation

Modified PPF method [130] was used to produce alginate microcapsules with spherical shape and uniformity. 2.0% (w/v) and 1.5% (w/v) alginate solutions were prepared by dissolving sodium alginate (Protanal® SF 120 alginate, FMC, Philadelphia, PA) in dH_2O. The pH of the solution was set to 7.2 for biological use. Isolated islets, which went through overnight recovery at 37°C with 5% CO_2, were collected from the cell medium (RPMI 1650 with 10% FBS and 1% PS) by centrifugation (3,000 rpm for 5 min) and gently dispersed in 1.5% (w/v) alginate solution. 2.0% (w/v) alginate solution and 1.5% (w/v) alginate solution containing islets (8,000 islets/ml) were sent through the outer and inner tube of the dual coaxial nozzle, respectively, with two separate syringe pumps. The dual coaxial nozzle with applied flow rates of 2.3 and 4.6 ml/min, respectively, for inner and outer nozzle from the two syringe pumps produced a jet consisting of outer shell and inner core containing islet suspension. An applied modulation frequency to the PPF system generated an acoustic excitation which broke the jet into uniformly sized microdroplets which were immediately collected into a gelling solution (50 mM $CaCl_2$, 1 mM $BaCl_2$, and 0.05% (v/v) Tween 20 in dH_2O, pH 7.2) under gentle stirring. The average microcapsule production time with PPF system was 5 min. After 5 min gelling time, the crosslinked alginate microcapsules containing islets were

washed with PBS and re-dispersed in RPMI-1640 with 10% FBS and 1% PS, and incubated at 37 with 5% CO_2.

3.2.2. *In vitro* islet evaluation after encapsulation

Each aliquot (1 mL) of alginate microcapsules containing islets was transferred to two different calibration grids and used for *in vitro* assessment with DTZ and SYTO-green/EB staining. The procedures were the same as described in previous chapter. Optical microscope was used to identify DTZ-stained islets and fluorescence microscope was used to qualitatively assess viability of encapsulated islets.

3.2.3. Statistical analysis

All data are expressed as mean ± standard deviation (SD). Data comparisons were performed by student t-test. Differences with $p < 0.05$ were considered statistically significant.

3.3. Results and Discussion

3.3.1. Characterization of alginate microcapsules

Alginate, which can be formed into micro-scale hydrogel, is one of the biomaterials that already has been used for numerous biomedical applications due to its favorable properties such as biocompatibility and fast gelation process [123]. This study employed modified PPF method to produce uniformly sized alginate hydrogels for rapid islet encapsulation. The dual coaxial nozzle and the acoustic excitation generated from the PPF system produced alginate microdroplets with a core/shell structure as illustrated

in Figure 4. The generated acoustic excitation can produce microdroplets with the rate of > 1,000 drops/sec which allows rapid production process with high throughput, therefore making PPF method favorable for islet encapsulation. The diameter of the alginate microdroplets can be determined by the flow rates of inner core solution and outer shell solution along with the molecular weight and the concentration of the feeding solution [131]. PPF method produced uniform alginate microcapsules with an average diameter of 500 μm using the dual coaxial nozzle with the inner flow rate of 2.3 ml/min and outer flow rate of 4.6 ml/min (1:2 ratio). 500 μm size alginate microcapsules were chosen for this experiment to accommodate all sizes of isolated porcine islets which usually ranged from 50 to 250 μm. The semi-permeable barrier of the alginate microcapsule, which is illustrated in Figure 5(a), is designed to protect encapsulated islets from immune cells while letting islets survive and function for a prolonged period of time [132].

3.3.2. *In vitro* islet evaluation after encapsulation

The morphology of encapsulated islets remained to be spherical and structurally intact which indicates that no severe physical damage was done during the encapsulation process. DTZ staining further confirmed that insulin-secreting beta cells were present in the islet clusters with rigid surface barrier as shown in Figure 5(b). The core of the alginate microcapsules, which consisted of relatively low alginate concentration (1.5%) and lower crosslinking density than the outer shell, had more flexible environment to safely secure the encapsulated islets. Additionally, the outer shell of the microcapsules with relatively high alginate concentration (2.0%) and crosslinking density prevented any islets from protruding from the microcapsules. Cell protrusion could lead to rejection

followed by necrosis and graft failure, therefore, it is very important to avoid it in order to achieve immunoisolation in microencapsulation [133, 134]. Figure 6 shows the optical and fluorescence micrographs of the alginate microcapsules containing islets where the SYTO-green and EB staining was used to visually and qualitatively assess the viability of encapsulated islets. There was no significant decrease in viability observed after the encapsulation due to fast encapsulation procedure with modified PPF method.

3.4. Conclusion

The present method using PPF system demonstrated a fast production of uniform alginate microcapsules containing islets with control in microcapsule diameter. The islets showed no significant damage after the encapsulation which further supports the feasibility of using PPF method for future porcine islet xenotransplantation applications. Future *in vivo* study needs to be done to further demonstrate the immunoisolation capability of microencapsulation strategy using PPF system.

3.5. Figures

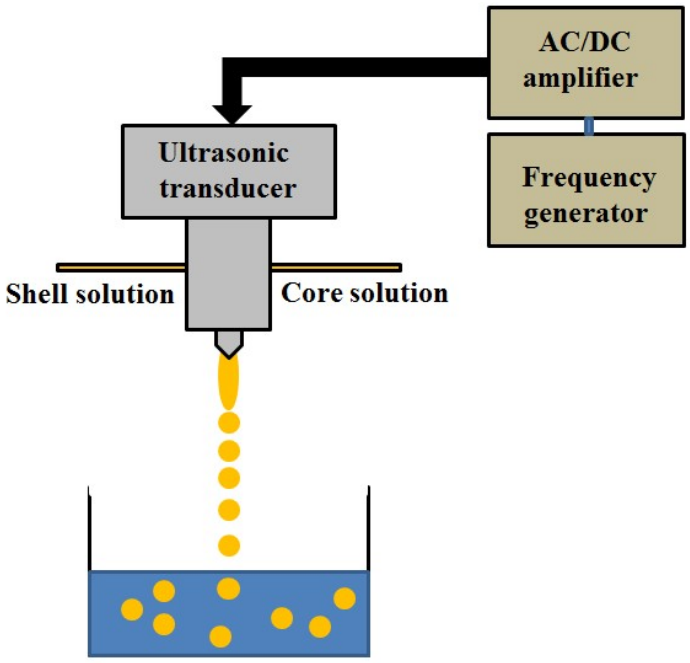

Figure 4. Schematic of PPF system for live cell encapsulation

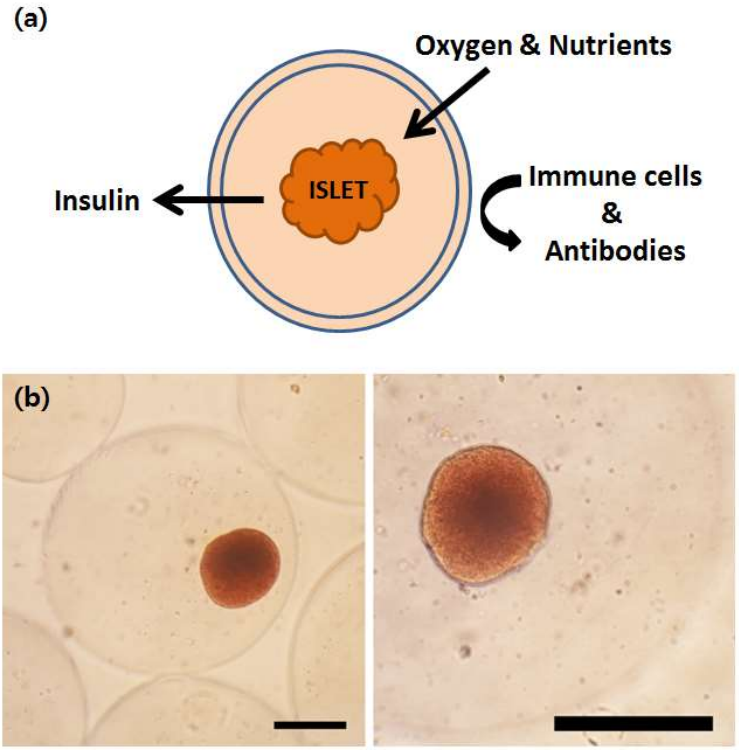

Figure 5. (a) A schematic of islet encapsulation with semi-permeable alginate microcapsules and (b) optical micrograph of islet encapsulated in alginate microcapsule after DTZ staining. Scale bar = 200 μm.

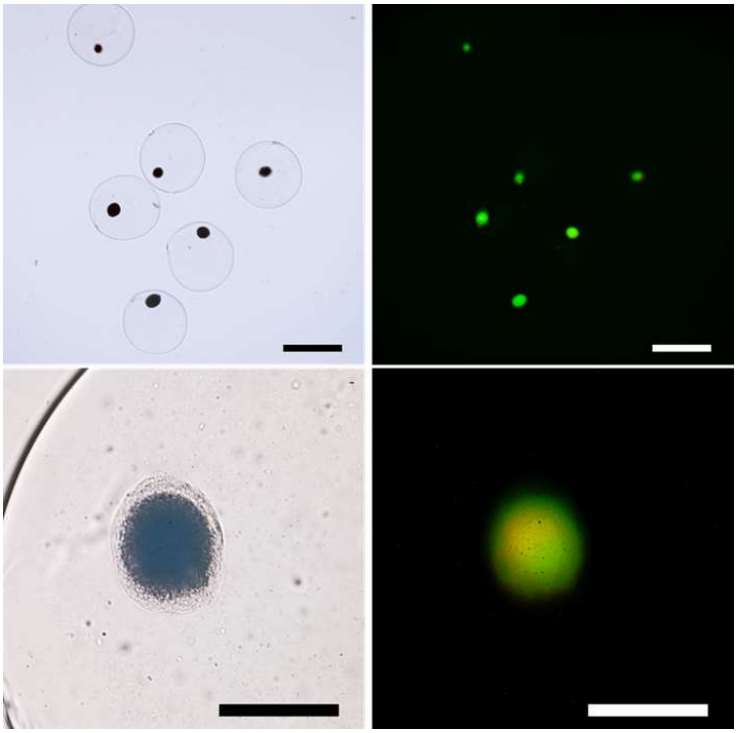

Figure 6. Optical (left) and fluorescence (right) micrographs of encapsulated islets. SYTO-green staining and ethidium bromide (EB) staining were used to visualize islet viability after encapsulation. SYTO-green (green) stains viable islets and EB (red) stains dead islets. Scale bar = 500 μm (top) and 200 μm (bottom).

CHAPTER 4

CONCLUSION AND FUTURE WORK

Microencapsulation technology has been widely utilized in various fields of biotechnology, especially in the medical and pharmaceutical industry. In order to enhance the efficiency and efficacy of using microencapsulation in clinical trials, it is important to have control in the following key aspects: microcapsule size and uniformity control, permeability, prevention of cell protrusion, reproducibility, and throughput. Precision particle fabrication (PPF) system was previously used to produce micro-scale capsules with many synthetic and natural biomaterials such as PLGA, PCL, PEG-PCL copolymer, gelatin, chitosan and alginate for different biotechnology applications. Alginate, which has good biocompatibility and fast gelling mechanism, is still widely used in various biomedical applications, especially in the field of xenotransplantation of porcine islets. PPF system's single step process using coaxial dual nozzle and acoustic excitation could easily handle highly viscous alginate solution to produce monodisperse microcapsules with core/shell structure and porosity. The size of the microcapsule can be controlled using different flow rates, frequency of the acoustic excitation, and alginate concentration. The PPF system also allows high throughput by producing alginate microcapsules at a rate of >1,000 drops/sec.

This study first reviewed and established the conventional method of isolating and purifying porcine islets using 5-6 month old commercial breed pigs provided by the University of Illinois slaughterhouse. Securing sufficient amount of isolated porcine islets

is the first crucial step towards islet xenotransplantation as it could solve the problem of donor shortage.

Furthermore, this study demonstrated the feasibility of using PPF method in microencapsulation of porcine islets for the treatment of type 1 diabetes. Monodisperse alginate microcapsules with a size of 500 μm were produced to encapsulate viable porcine islets which were isolated and purified the day before the encapsulation. The results showed viable and functioning porcine islets after the isolation and purification. The core/shell structure of the alginate microcapsules was designed to safely protect islets from host's immune reactions, therefore achieving immunoisolation. Due to the fast and easy one step process of PPF system along with the fast gelation process of alginate, the porcine islets after the encapsulation still remained viable without any severe damage.

The future work will be to facilitate and monitor prolonged viability and function of encapsulated islets *in vitro* and *in vivo*. Additional future study will use PPF system to co-encapsulate islets and various active agents, with the capability to synergistically support islets, to observe improved survivability and function of encapsulated islets for prolonged period of time.

CHAPTER 5

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